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Mapping of Functional Regions of *Clostridium perfringens* Type A Enterotoxin

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Studies were conducted to allow construction of an initial map of the structure-versus-function relationship of the *Clostridium perfringens* type A enterotoxin (CPE). Removal of the N-terminal 25 amino acids of CPE increased the primary cytotoxic effect of CPE but did not affect binding. CPE sequences required for at least four epitopes were also identified.

The localization of functional activities to defined regions of toxin molecules has been reported for several intracellularly active toxins (1, 9, 10, 25). A similar detailed understanding of structure-function relationships does not yet exist for the large family of membrane-active bacterial toxins. *Clostridium perfringens* enterotoxin (CPE, 35 kDa), a membrane-active toxin with a multistep action (4, 8, 14–20, 26, 27), is responsible for symptoms associated with *C. perfringens* type A food poisoning (16). Early steps in enterotoxin action include specific binding of CPE to a proteinaceous receptor(s), insertion of toxin into mammalian plasma membranes, and formation of a 160-kDa complex between CPE and mammalian membrane proteins. CPE-challenged cells then develop extensive membrane permeability alterations for small molecules (e.g., ions and amino acids) which cause secondary effects (e.g., inhibition of macromolecular synthesis) resulting in cell death.

Although still incomplete, the understanding of the CPE structure-function relationship is relatively advanced for a membrane-active toxin (2, 3, 5–7, 26, 28) and may serve as a model system for these toxins. We now report that a 32.5-kDa fragment (termed CPE_{26-319}) produced by limited trypsinization of CPE (2, 3, 26) has two- to threefold more cytotoxic activity than native CPE and that this increased CPE_{26-319} activity is not due to enhanced binding characteristics. Epitope mapping studies of the enterotoxin are also presented. This new information is combined with previous results to construct a molecular map of CPE activities.

CPE was purified and radioiodinated as described previously (18, 21). CPE fragments used in this study are shown diagrammatically in Table 1. Trypsin-derived CPE_{26-319} was prepared by limited treatment of either CPE or ^{125}I-CPE (0.5 to 1.0 mg/ml) with immobilized trypsin (Sigma) as described previously (2, 3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1) indicates that the presumed CPE_{26-319} produced by this method migrated at 32.5 kDa. This result is consistent with previous studies (2, 3, 22), which indicated that limited trypsinization removes the 25 N-terminal amino acids of enterotoxin. Additionally, amino acid sequencing of the amino terminus of the presumed CPE_{26-319} preparation identified the single sequence TPIN ITNNN expected for CPE_{26-319} (22). Genetically produced CPE fragments in the *Escherichia coli* lysates used in this study were from frozen preparations characterized previously (4–6) (Table 1). The $CPE_{290-319}$ synthetic peptide and a negative control synthetic peptide used in this study (Table 1) were also from preparations characterized previously (5). Protein and synthetic peptide concentrations were determined as described previously (5, 12).

Previous reports (2, 3, 22, 26) have indicated that the CPE_{26-319} fragment causes two- to threefold more secondary effects (i.e., erythemal activity and protein synthesis inhibition in Vero cells) when compared with equimolar amounts of native CPE. This trypsin enhancement of biological activity could be physiologically important since enterotoxin may be proteolytically modified in the small intestine by intestinal proteases, possibly resulting in increased CPE potency during food poisoning (3). However, the mechanism behind this trypsinization enhancement is not known. Therefore, studies were conducted to determine whether CPE_{26-319} has enhanced primary (i.e., alteration of small molecule permeability) as well as secondary CPE effects and to compare the binding characteristics of CPE_{26-319} with those of native CPE.

We now report that, on an equimolar basis, ^{125}I-CPE_{26-319} caused two to three times more ^{86}Rb release (15, 19, 24, 26) than ^{125}I-CPE. For example, 29 nM ^{125}I-CPE_{26-319} caused 51.2% ± 2.2% of maximal ^{86}Rb release while 29 nM ^{125}I-CPE caused only 19.3% ± 8.8% of maximal ^{86}Rb release. These results demonstrate that limited trypsinization increases the primary CPE cytotoxic effect (8). This strongly suggests that CPE_{26-319} shares a similar, although enhanced, mechanism of action with native CPE and validates the use of CPE_{26-319} as a probe for elucidating the CPE structure-function relationship. This is important since a 16-kDa fragment produced (2) by trypsin treatment of CPE in the presence of SDS has an artifactual toxic effect (i.e., inhibition of protein synthesis in vitro). This effect is not shared by native CPE, which is membrane active and does not enter the cytoplasm (16).

Binding experiments were performed to determine whether the enhanced cytotoxicity of trypsin-generated CPE_{26-319} was due to an increase in specific binding of CPE_{26-319} as compared to that of native CPE. Although a binding region has been localized to the C terminus of native CPE (5), removal of the CPE N-terminal region by trypsin treatment conceivably could improve binding characteristics

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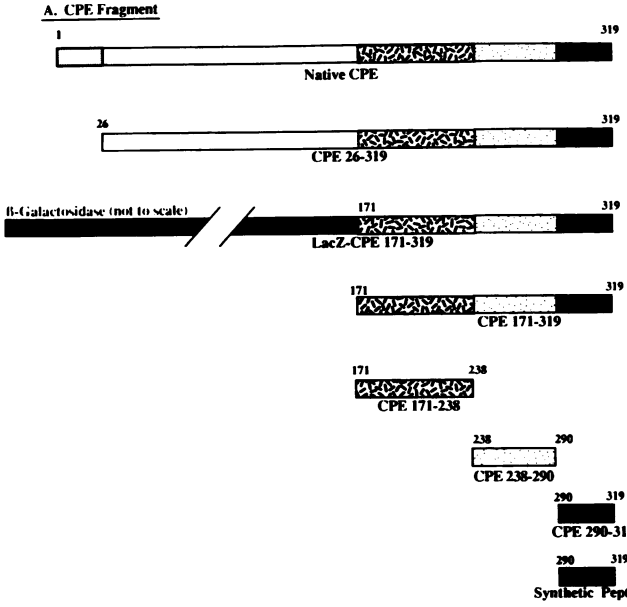
TABLE 1. Properties and descriptions of CPE fragments and antibodies used in this study

A. CPE Fragment



Description/Source (Ref)	Abbreviated Name	Receptor-Binding Activity	Cytotoxicity
purified native CPE (21)	CPE_{1-319}	+	+
large fragment from trypsin-treated CPE (2,3)	CPE_{26-319}	+	++
lysates from *E. coli* lysogenized with Lambda pH161 which produces a lacZ-$CPE_{171-319}$ fusion protein (6)	lacZ-$CPE_{171-319}$	-	-
lysates from *E. coli* transformed with pPH300 which encodes $CPE_{171-319}$ (6)	$CPE_{171-319}$	+	-
lysates from *E. coli* transformed with pPH400 which encodes $CPE_{171-238}$ (5)	$CPE_{171-238}$	-	-
lysates from *E. coli* transformed with pPH500 which encodes $CPE_{238-290}$ (5)	$CPE_{238-290}$	-	-
lysates from *E. coli* transformed with pPH600 which encodes $CPE_{290-319}$ (5)	$CPE_{290-319}$	+	-
synthetic peptide corresponding to $CPE_{290-319}$ (5)	synthetic peptide	+	-

B. Antibodies

Antibody:	Rabbit Polyclonal anti-CPE (RPC)	MAb 1G7	MAb 3C9	MAb 11H8	MAb 14A7	MAb β-gal	Neg MAb	Normal Rabbit Serum (NRS)
Origin and Descriptions (Ref):	from rabbits immunized with native CPE (28)	murine hybridoma from native CPE immunization (28)	murine hybridoma from native CPE immunization (28)	murine hybridoma from native CPE immunization (28)	murine hybridoma from native CPE immunization (28)	MAb to β-galactosidase (Promega)	MAb to epitope on oligodendrocyte cell surface (23)	Cappel Laboratories
Neutralizes CPE Cytotoxicity:	+	-	+	-	-	-	-	-
Blocks CPE Binding:	+	-	+	-	-	-	-	-

by producing distal binding-enhancing conformational changes in CPE_{26-319}. However, the results shown in Fig. 2 indicate that the increased cytotoxicity of CPE_{26-319} is not due to enhanced binding since there were no differences between the specific binding rates or the specific binding levels of ^{125}I-CPE and ^{125}I-CPE_{26-319}. This indicates that trypsinization increases one or more postbinding events in CPE action.

To continue the identification of important regions of the CPE molecule, epitope mapping studies were also conducted (Fig. 3). The origin and properties of antibodies used in this study are described in Table 1. Rabbit polyclonal antisera were absorbed against control strain *E. coli* RR1 before use. Monoclonal antibodies (MAbs) to CPE recognize distinct epitopes and were purified from ascites fluid as described previously (28). By immuno-dot-blot analysis, native CPE showed immunoreactivity with rabbit polyclonal anti-CPE serum (RPC) and all anti-CPE MAbs but not with normal rabbit serum or with negative control MAbs to an oligodendrocyte epitope or to an epitope on β-galactosidase (Fig. 3). If CPE was denatured by boiling in the presence of SDS prior to immuno-dot-blot analysis, RPC and all anti-CPE MAbs except MAb 1G7 retained immunoreactivity (data not shown). This suggests that the epitopes recognized by MAbs 3C9, 11H8, and 14A7 are linear, while the epitope recognized by MAb 1G7 is conformational (13).

There were various patterns of immunoreactivity for different CPE fragments (Fig. 3). CPE_{26-319} showed immunoreactivity identical to that of native enterotoxin except that it was not recognized by MAb 1G7, strongly suggesting that presentation of the MAb 1G7 epitope requires extreme N-terminal CPE sequences. *E. coli* lysates containing either a *lacZ* $CPE_{171-319}$ fusion protein or the $CPE_{171-319}$ fragment free from β-galactosidase reacted with both RPC and MAb 3C9. However, MAb 14A7 was able to recognize the free $CPE_{171-319}$ fragment but not the fusion protein, indicating that (i) presentation of the MAb 14A7 epitope requires some sequences in the C-terminal half of enterotoxin and (ii) the MAb 14A7 epitope is sterically blocked in the fusion protein by the large β-galactosidase moiety. MAb 11H8 recognized CPE_{26-319} but not $CPE_{171-319}$, indicating that sequences between amino acids 26 and 171 are required for the presentation of the MAb 11H8 epitope. None of the antibodies used in this study reacted with *E. coli* lysates from strains which carry expression plasmids encoding $CPE_{171-238}$ or $CPE_{238-290}$. These fragments should be expressed because

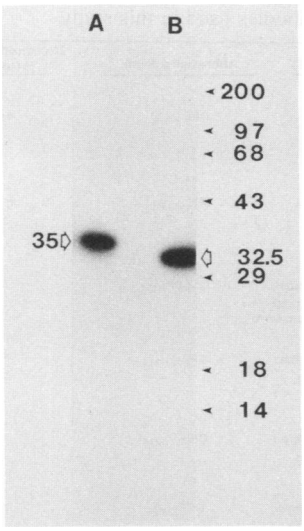


FIG. 1. SDS-PAGE autoradiograph of ^{125}I-CPE$_{26-319}$ preparation. ^{125}I-CPE$_{26-319}$ was prepared, and the large CPE fragment was isolated by gel filtration as described previously (2, 3, 26). ^{125}I-CPE (lane A, 15,000 cpm) or ^{125}I-CPE$_{26-319}$ (Lane B, 15,000 cpm) was analyzed by SDS-PAGE on 12% acrylamide by using the buffer system of Laemmli (11). The calculated molecular weights of both species are indicated by the number adjacent to the open arrow. All molecular sizes shown are in kilodaltons.

these plasmids contain the correct *cpe* gene inserts in the proper orientation and reading frame for expression (5). However, immunoreactivity might be expected with one or both of these lysates since MAb 14A7 recognizes a linear epitope in CPE$_{171-319}$ but does not react with CPE$_{290-319}$. This suggests either that CPE$_{171-238}$ and CPE$_{238-290}$ are degraded rapidly after synthesis or that presentation of the

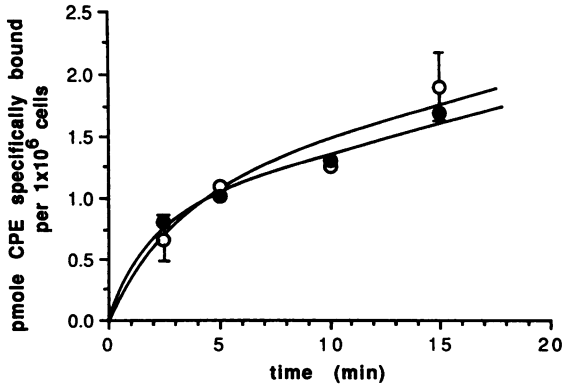


FIG. 2. Comparison of ^{125}I-CPE$_{26-319}$ specific binding with ^{125}I-CPE specific binding to Vero cells. Vero cells (10^6 per plate) were incubated for the specified times at 37°C with 29 nM ^{125}I-CPE$_{26-319}$ (●) or 29 nM ^{125}I-CPE (○) in the presence (representing nonspecific binding) or absence (representing total binding) of a 50-fold excess of unlabeled CPE (18). After processing and counting sample radioactivity in a gamma counter (18), specific binding was calculated as the difference between total binding samples and nonspecific binding samples. Binding results were corrected for differences in specific activity between ^{125}I-CPE$_{26-319}$ and ^{125}I-CPE. Results shown are means ± standard errors from three experiments with triplicate samples at each time point. Points without error bars had standard errors too small to depict.

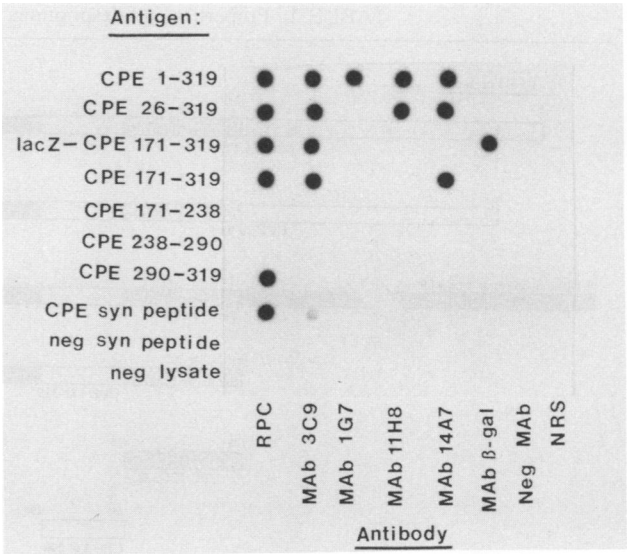


FIG. 3. Immuno-dot-blot analysis of CPE epitopes. Antigens (500 ng of synthetic peptide or 25 μg of protein of *E. coli* lysate) were immobilized on nitrocellulose and reacted with antibodies (a 1/3,000 dilution of RPC or normal rabbit serum or a 1/1,000 dilution of anti-CPE MAb or negative control MAb) as described previously (5), except ^{125}I-protein A (50 nCi/ml) was substituted for the secondary antibody-enzyme conjugate used previously (5). Immunodot blots were exposed to X-ray film for 24 to 48 h at −70°C by using an intensifying screen. Negative control lysate is from an *E. coli* RR1 strain lacking a CPE insert, while negative control synthetic peptide corresponds to residues 46 through 61 of hen egg lysozyme (5). See Table 1 for description of other antigens and antibodies. Identical immuno-dot-blot results were obtained if preimmune serum was substituted for normal rabbit serum (not shown).

MAb 14A7 epitope is disrupted in the construction of plasmids encoding these small fragments.

In Fig. 3, both the CPE$_{290-319}$ synthetic peptide and *E. coli* lysates containing recombinant CPE$_{290-319}$ were recognized by RPC. However, only the CPE$_{290-319}$ synthetic peptide was recognized by MAb 3C9. Two experimental observations suggest that the inability of MAb 3C9 to react with CPE$_{290-319}$ lysate is real and not due to an insufficient amount of CPE$_{290-319}$ in the lysate for detection to occur. First, densitometry comparisons of RPC reactivity with CPE$_{290-319}$ lysate and with CPE$_{290-319}$ synthetic peptide suggest that approximately equal amounts of the total CPE$_{290-319}$ epitope(s) were present in these samples. Second, while RPC blocked the specific binding (measured as described previously [5]) of recombinant CPE$_{290-319}$ to brush border membranes, MAb 3C9 did not affect recombinant CPE$_{290-319}$ binding (data not shown). Therefore, these results suggest that the nine vector-encoded amino acids added to recombinant CPE$_{290-319}$ (5) may disrupt presentation of the MAb 3C9 epitope.

The weak reactivity of MAb 3C9 with the CPE$_{290-319}$ synthetic peptide compared to that with native CPE was reproducible in three repetitions of the experiment shown in Fig. 3. Densitometry of the MAb 3C9 immunodots indicated that the CPE$_{290-319}$ synthetic peptide immunodot had only 8% of the intensity of the native CPE dot blot. A competitive enzyme-linked immunosorbent assay (ELISA) (Fig. 4) confirms that MAb 3C9 recognizes native CPE better than synthetic peptide since it was necessary to preincubate MAb

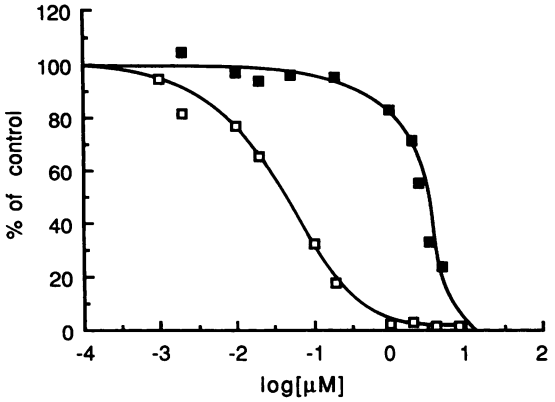


FIG. 4. Competitive ELISA analysis of CPE versus $CPE_{290-319}$ synthetic peptide binding to MAb 3C9. Specified concentrations of native CPE (□) or $CPE_{290-319}$ synthetic peptide (■) were preincubated with 0.06 μg of MAb 3C9 per ml for 30 min at 37°C. The preincubation mixtures were then added for 2 h to ELISA plates coated with 0.5 μg of native CPE, and ELISA analysis was performed as described previously (28). Optical density was read at 405 nm. Results are expressed as percent of control optical density (i.e., no competitor present) versus log concentration (in micromoles per liter). Data from a representative experiment are shown. This experiment was performed three times with similar results.

3C9 with 80-fold more synthetic peptide than native CPE to obtain similar 50% reductions in subsequent ELISA optical densities.

Collectively, these results suggest that there are at least two epitopes between amino acids 290 and 319 of native CPE. One epitope (termed RPC-1) is present in both $CPE_{290-319}$ synthetic peptide and recombinant $CPE_{290-319}$ and is strongly recognized by RPC. The second epitope (termed the MAb 3C9 epitope) is a linear epitope presented in the $CPE_{290-319}$ synthetic peptide but not in recombinant $CPE_{290-319}$. This epitope is recognized by MAb 3C9 and possibly by non-RPC-1 antibodies in RPC. Since MAb 3C9 is a high-affinity MAb (28) which recognizes native CPE more strongly than the $CPE_{290-319}$ synthetic peptide, this difference suggests that sequence information outside the 30 C-terminal CPE residues also may contribute to optimal presentation of the MAb 3C9 epitope.

From these experiments and previous studies (2–7, 22, 28), it is possible to construct an initial molecular map of functional regions on the CPE molecule (Fig. 5). The current studies suggest that (i) there are at least five distinct CPE epitopes, (ii) sequences required for presentation of the four nonoverlapping epitopes recognized by the anti-CPE MAbs used in this study are distributed throughout the enterotoxin, and (iii) there are two epitopes associated with the extreme C terminus of enterotoxin. Since RPC blocks the binding of both the recombinant $CPE_{290-319}$ fragment and the $CPE_{290-319}$ synthetic peptide (5), this suggests that both the RPC-1 and MAb 3C9 epitopes are neutralizing epitopes. Since neither the recombinant fragment nor the synthetic peptide corresponding to $CPE_{290-319}$ is cytotoxic (5), these results also strongly suggest that the $CPE_{290-319}$ region might be used safely to obtain CPE-neutralizing antibodies in vivo. This hypothesis currently is being tested and should contribute to construction of a CPE vaccine prototype.

The functional map also incorporates our CPE_{26-319} data with previous results (4, 5) (i) to localize CPE binding to the extreme C-terminal 30 amino acids and (ii) to indicate the presence between CPE residues 26 and 171 of critical sequences required for insertion and cytotoxicity. It remains unresolved whether there are any neutralizing epitopes outside the $CPE_{290-319}$ region. The N-terminal region of enterotoxin does not seem to be required for any function related to CPE action. It is not a signal polypeptide since CPE is not a secreted protein but is released upon mother-cell lysis during sporulation (16, 21). The increased postbinding cytotoxicity of CPE_{26-319} may result from a favorable conformational change in the nearby cytotoxicity region when the N-terminal region is removed. A similar proteolytic activation of enterotoxin could theoretically occur in the intestine during food poisoning. However, direct evidence for this effect is still lacking.

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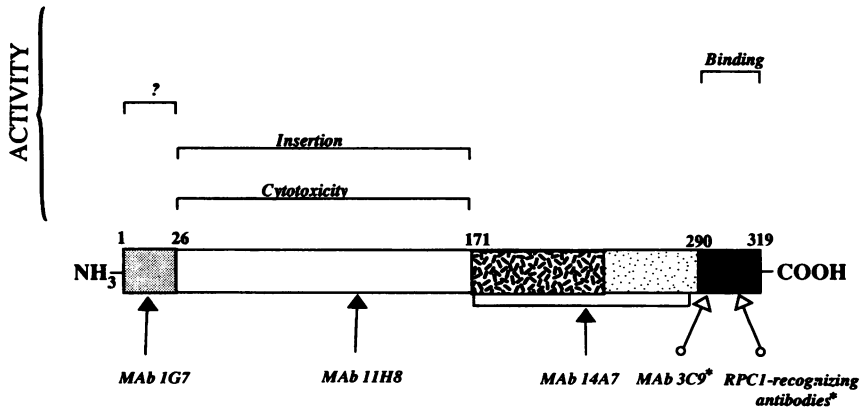

FIG. 5. Map of CPE functional regions. CPE regions required for insertion, cytotoxicity, and binding are shown (present study) (4–7). CPE sequences shown (present study) to be required for presentation of the epitopes recognized by MAb 1G7, MAb 11H8, and MAb 14A7 are indicated by solid arrows, while CPE regions shown (present study) to contain all or part of the MAb 3C9 and the RPC-1 epitopes (see text) are indicated by open arrows. MAb 11H8, MAb 14A7, and MAb 3C9 recognize linear epitopes, while the MAb 1G7 epitope is conformational (see text). Asterisks designate antibodies which neutralize biological activity.

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